LWT - Food Science and Technology 60 (2015) 743–750

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Validation of nutraceutical properties of honey and probiotic potential of its innate microflora

S. Benazir Begum, R. Raja Roobia, M. Karthikeyan, R.M. Murugappan*

Department of Zoology and Microbiology, Thiagarajar College, Madurai 625 009, Tamil Nadu, India

ARTICLE INFO

Article history:
Received 5 June 2014
Received in revised form
8 October 2014
Accepted 10 October 2014
Available online 27 October 2014

Keywords:
Honey
Nutraceuticals
Gluconobacter oxydans
Probiotics
Siderophore

ABSTRACT

Honey extracted from the beehives was found to be acidic (pH 4.7) with moderate moisture (17.27%), low hydroxymethylfurfural (HMF) level (4.62 mg/kg) and ash content (0.46%). A linear positive relationship was observed between the honey concentration and DPPH (2, 2 diphenyl-1-picrylhydrazyl) radical scavenging activity. Energy dispersive x-ray spectrum (EDAX) analysis of the honey revealed the presence of essential elements like K, Ca, Mn and Fe. The high R^2 values obtained with dose response plot inferred the inhibitory efficiency of honey toward different bacterial pathogens. *Gluconobacter oxydans* isolated from honey was found to possess probiotic properties with siderophorogenic potential. Siderophore produced by *G. oxydans* was found to be of trihydroxamate nature and formed hexadentate ligands with Fe^{3+} ions. Fourier-transform infrared (FTIR) spectra revealed the trihydroxamate nature of the siderophore produced. Growth promotion activity of siderophore was ascertained by cross utilization studies. This study indicated *G. oxydans* as a potential probiotic bacterium bearing siderophoregenic attribute that confer health benefits to the host.

1. Introduction

Honey is a sweet, viscous, highly nutritive, natural liquid plant exudates gathered, modified and stored in the comb by *Apis* spp. Honey has been used as a promising source to prevent and cure various ailments for more than 4000 years (Basualdo, Sgroy, Finola, & Mariola, 2007). Supported by cultural and traditional practices, anecdotal reports encourage feeding of newborn babies with honey as a dietary supplement (Ajibola, Chamunorwa, & Erlwanger, 2012). Ayurvedic physicians use honey as a vehicle of medicines and recommend it to meet out the immediate calorie requirement for the patients.

The quality of honey is determined by its sensorial, chemical, physical and microbiological characteristics. Generally, honey is acidic in nature (pH 4) and composed mainly of sugars (80%) and water (17%). Various enzymes, acids and minerals constitute the remaining 3% (Kahraman, Buyunkunal, Vural, & Altnatmaz, 2010). The high sugar and low-water content suppress the growth of bacteria and contribute to the stability of the product. The presence of a small number of bacterial species in the unpasteurized natural honey (NH) comprises its innate microflora (Tsekoura et al., 2006). Most of the innate microflora are of probiotics and confer health benefits to the host (Ajibola et al., 2012).

One of the beneficial effects of probiotics is cholesterol reduction either by assimilation or deconjugation of bile salts. Deconjugated bile salts are less soluble than conjugated bile salts, resulting in lower absorption and elimination in the feces (Suvarna & Boby, 2005). Other functional properties used to characterize an ideal probiotic are production of antimicrobial compounds, adhesion to gut tissue, resistance to antibiotics, absence of hemolytic activity, ability to tolerate acid and bile salts (Saarela, Mogensen, Fonden, Matto, & Mattila-Sandholm, 2000).

The most important property of honey is its inhibitory activity against bacterial pathogens. The bactericidal activity of honey has been attributed by its acidity, increased osmolarity, production of hydrogen peroxide, presence of phenolic acids, lysozyme, flavonoids etc., (Voidarou et al., 2011).

Further, honey serves as a rich source of iron. Iron is inevitable to all forms of life and required for a number of biological reactions like reduction of oxygen for the synthesis of ATP, reduction of ribotide precursors of DNA, for the formation of heme, detoxification of oxygen radicals etc., (Winkelmann, Schmid, Nicholson, Jung, & Colquhoun, 2002). To avail the iron present in the honey, innate microflora produces low molecular weight (<1000 D), multidentate iron chelating organic ligands known as siderophores. Ahire, Patil, Chaudari, and Chincholkar (2011), reported that siderophore synthesis is testimonial to the probiotic properties of the organism.

* Corresponding author. Tel.: +91 9443918665 (mobile).
E-mail address: cmmbmak@gmail.com (R.M. Murugappan).

http://dx.doi.org/10.1016/j.lwt.2014.10.024
0023-6438/

Though, physico-chemical status and antimicrobial properties of honey have been a subject of extensive analysis, a more comprehensive understanding of innate microbial flora is needed to unravel the therapeutic properties. Therefore, the present study is intended to study the physico-chemical parameters, probiotic and siderophoregenic potential of innate microflora of the honey extracted from the beehives. The outcome of this work will help to understand the usefulness of honey in clinical practice against bacterial infection and its nutritional value as a food supplement.

2. Materials and methods

2.1. Honey sample

The study was carried out with natural, untreated and unpasteurized honey freshly harvested from the bee hives of *Apis indica* collected from the foot hills of Western Ghats (latitude 10°21′N and longitude 78°35′E), Tamil Nadu, India. The sample was collected by squeezing the comb, filtered and stored under aseptic conditions at 5 °C until further analyses. The honey collected for analyses is of multifloral origin.

2.2. Physico-chemical properties of honey

The sample was analysed for pH, ash, moisture, acidity, sugars and hydroxyl methyl furfural (HMF) following Association of Official Analytical Chemists (AOAC) methods (1990). Electrical conductivity was measured as described by Bogdanov, Martin, and Lullmann (1997). Viscosity of honey was calculated using Ostwald's viscometer following the method of Akoh (1991). Three replicates were maintained for all the experiments in this study unless otherwise represented.

2.2.1. EDAX analysis

Elemental composition of the honey sample was analyzed using Field emission scanning electron microscope (FESEM) equipped with Energy dispersive x-ray analysis (JEOL-JSM-6701F).

2.2.2. Assessment of antimicrobial and antioxidant activity

Susceptibility of bacterial pathogens (*Staphylococcus aureus* (MTCC 2940), *Escherchia coli* (MTCC 739), *Micrococcus luteus* (MTCC106), *Pseudomonas aeruginosa* (MTCC 424), *Bacillus subtilis* (MTCC 1427) and *Salmonella typhi* (MTCC 733)) to different concentration (20, 40, 60, 80 and 100% v/v) of honey was determined following agar well diffusion method (Cappucino & Sherman, 2006). Dose-response influence of honey on different pathogenic strains was calculated using prism software (ver. 6.04). Antioxidant potential of honey was measured by DPPH (2, 2-Diphenyl-1-pirylhydrazyl) method (Ferreira, Aires, Barreira, & Estevinho, 2009). Percentage of free radical scavenging activity of honey was calculated using the following equation:

$$\text{Radical scavenging activity } (\%) = (A_{DPPH} - A_s/A_{DPPH}) \times 100$$

where, A_{DPPH} is the absorbance of DPPH solution and A_s is the absorbance of sample at different concentration.

2.3. Isolation and identification of bacteria

Bacteria present in the honey were isolated by spread plate technique using tryptic soy agar (Cappucino & Sherman, 2006). Hemolytic activity of the isolates was screened using brain heart infusion agar supplemented with sheep blood (5%). Non-hemolytic isolate was selected and identified based on morphological and biochemical characteristics as described by Bergey's manual of determinative bacteriology (Brenner, Kreig, & Staley, 2005). For molecular characterization, genomic DNA was extracted from the isolate and 16S rDNA was amplified using the forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 5′-ACGGCTACCTTGTTACGACTT-3′. The 16S rRNA gene sequence of the isolate was compared with related gene sequences available at NCBI. Selected sequences were aligned in bio-edit and phylogenetic analysis was performed using molecular evolutionary genetics analysis software package (MEGA ver. 4.0).

2.4. Screening for probiotic properties

2.4.1. Acid and bile salt tolerance

Probiotic microorganism must overcome physical and chemical barriers in the gastrointestinal tract. Therefore, in this study acid tolerance property of the isolate was determined following the procedure described by Erkkila and Petaja (2000). The isolate was grown in tryptic soy broth for 24 h at 30 °C. After incubation, cells were harvested by centrifugation at 10,000 rpm for 10 min, washed, re-suspended in 1 ml of sterile phosphate buffered saline (PBS) at different pH for various time intervals (0, 60, 120, 180 min). Later the mixture was transferred to fresh tryptic soy broth and incubated at 30 °C. The growth of bacteria was measured at A_{620nm} after 24 h of incubation and the percent survival of strain to different pH was calculated. Data were statistically analyzed using analysis of variance (ANOVA).

The bile salt tolerance of the isolate was determined as per Bilige et al. (2009). Tryptic soy broth (100 ml) supplemented with different concentration of bile salt (wt/vol. oxgall) was prepared and inoculated with one ml (3×10^7 cells ml^{-1}) of the isolate and incubated at 30 °C. Growth of bacteria was measured (A_{620nm}) at different time intervals and the percent survival of the isolate was calculated.

2.4.2. Cholesterol assimilation assay

The ability of isolate to assimilate cholesterol was assessed as per the procedure described by Bilige et al. (2009) with certain modifications. One ml of bacterial suspension (3×10^7 cells ml^{-1}) was inoculated in 100 ml of MRS broth containing fresh, filter-sterilized water soluble cholesterol (150 μg/ml) and bile salt (0.3%). The medium was incubated for different time durations at 30 °C and the cells were harvested by centrifugation at 10,000 rpm for 10 min. The growth of the isolate was determined in terms of micrograms protein per milliliter (Bradford, 1976). The residual cholesterol in the supernatant was determined by measuring the absorbance at 546 nm using spectrophotometer. The percentage assimilation of cholesterol was calculated.

$$\text{Cholesterol assimilation } (\%) = (B - C)/B \times 100$$

where, B represent the absorbance (A_{546}) of blank and C represent the absorbance (A_{546}) of cell supernatant.

2.4.3. Auto-aggregation, solvent adhesion and antibiotic susceptibility assay

Auto-aggregation and adhesion of bacterial cells to different solvents was calculated as per the procedure described by Del Re, Sgorbati, Miglioli, and Palenzona (2000) and Ahire et al. (2011) respectively. The isolate was grown in tryptic soy broth for 24 h at 30 °C. The cells were pelleted, washed twice with PBS (pH 7.3), resuspended in the same buffer to get an absorbance (660 nm) of 0.5 as A_0. For auto-aggregation assay, the bacterial suspension was incubated for different time durations and absorbance was measured (A_1). For solvent adhesion assay, 3 ml of cell suspension

was mixed with 1 ml of different solvents (xylene, chloroform and ethyl acetate) and incubated for 20 min. After incubation, the absorption was measured as A_1. The percentage of auto-aggregation and adhesion to different solvents was calculated as $(A_0 - A_1/A_0) \times 100$. The susceptibility of the isolate to different antibiotics was determined using standard antibiotic discs (Hi Media, Mumbai) on Muller Hinton agar.

2.5. Screening of siderophore production

Siderophore production in the isolate was determined by $FeCl_3$ test and chrome azurol sulphonate (CAS) assay (Schwyn & Neilands, 1987). Nature of siderophore produced was ascertained by Csaky, Arnow and Vogels assay (Payne, 1994). Siderophore was further distinguished into mono-, di- or trihydroxamates based on the absorption maxima and electrophoretic mobility (Jalal & Helm, 1990). A shift in λ-max as a function of various pH values was used to differentiate the ferric complexes of hydroxamate siderophore. The iron-binding property of siderophore was determined by the stable/unstable nature of the color of ferrate siderophore at different pH values.

2.5.1. Purification and characterization of siderophore

Siderophore produced by the isolate was purified using Amberlite XAD 400 resin and Sephadex LH-20 column as described by Sayeed and Chincholkar (2006) and lyophilized. Lyophilized siderophore sample was pelleted with potassium bromide (KBr) and subjected to FTIR spectroscopy (Shimadzu IR Affinity1, Japan) for determination of functional groups. Spectra were recorded in the range from 4000 to 400 cm^{-1}.

2.5.2. Growth promotion assay

Siderophore-mediated growth promotion was performed as suggested by Winkelmann et al. (2002). Fifty μl of siderophore auxotrophic bacterial culture was inoculated into an iron deprived MM9 medium (10 ml) containing 50 μl of filter sterilized natural honey. Five microliter of purified siderophore was added to the medium and incubated for 24 h. The growth of the siderophore auxotrophs with and without siderophore was determined in terms of micrograms protein per milliliter (Bradford, 1976).

3. Results and discussion

3.1. Chemical composition

Chemical composition of unpasteurized honey collected from the bee hives is depicted in Table 1. The sample was acidic (pH 4.7) with moderate moisture (17.27%) and low HMF level (4.62 mg/kg).

Table 1
Physico – chemical parameters of natural honey (NH).

Parameters	Units	NH
pH	–	4.75
Ash content	%	0.46 ± 0.08
Moisture content	%	17.27 ± 0.13
Free acidity	Meq/kg	17.53 ± 1.509
Lactic acidity	Meq/kg	5.24 ± 0.12
Total acidity	Meq/kg	22.9 ± 1.437
Color	–	Light amber
Viscosity	centipoise	3.1 ± 0.08
Electrical conductivity	ms/cm	0.46 ± 0.107
Hydroxy methyl furfural	mg/kg	4.62 ± 0.36
Carbohydrate composition		
Total sugar	g/100 g	78.79 ± 0.591
Reducing sugar	g/100 g	71.79 ± 1.498
Sucrose	g/100 g	7.543 ± 0.139

Data are given as mean ± standard error of three replicates.

The viscosity was found to be 3.1 centipoise. Cervanetes, Novelo, and Duch (2000) reported that the viscosity decreases as the water content increases. Free acidity, lactic acidity and total acidity of the honey sample were found to be 17.53 meq·kg^{-1}, 5.2 meq·kg^{-1} and 22.9 meq·kg^{-1} respectively. The physico-chemical values analyzed were within the acceptable international standards (Codex Alimentarius, 2001) and indicate the freshness, flavor and stability of the product. The ash content and electrical conductivity were closer to the permissible limits (0.6% and 0.8 ms·cm^{-1} respectively). Naman, Faid, and El-Adlouni (2005) reported that electrical conductivity value reflects the total mineral (ash) and acid content of the sample. Sugars the main component of honey was found to be 78.79 g/100 g. The mean value of sucrose concentration is in equilibrium with the maximum allowable limit (≤8%) indicating the early harvest.

3.1.1. EDAX analysis

Presence of essential elements in honey was determined by Energy dispersive x-ray spectrum (Fig. 1). The sharp peaks in the spectral image of EDAX represent the occurrence of essential elements like K, Ca, Mn and Fe in honey which is in agreement with Silva, Alves, Fernandes, and Muller (2013). The level of lead, zinc and copper were below the detectable range. Hamouda and Abouwarda (2011) reported that the elemental composition of honey varies with floral source, geographical location and climatic conditions. Elemental composition was used not only for quality assessment of honey but also for the determination of environmental contamination (Kamboj, Bera, & Nanda, 2013).

3.1.2. Antimicrobial activity

Dose–response curves obtained by plotting the log concentration of honey against the growth of different pathogens are shown in Fig. 2. High R^2 values obtained in the regression plot represent the inhibitory efficiency of honey. The percentage inhibition model provides a good linearization of the dose–response data for the tested pathogens. From the dose response analysis, it can be deduced that the honey was more effective against *E. coli* and *S. aureus* than other pathogens tested. Gulfraz et al. (2011) reported that the antimicrobial activity of honey could be attributed to several factors including osmotic effect, acidity and enzymatic generation of hydrogen peroxide.

3.1.3. Antioxidant activity

The DPPH (2,2 diphenyl-1-picylhydrazyl) has been extensively used to test the free radical scavenging ability of various food supplements. The chromogen radical DPPH was scavenged by the antioxidants present in the sample through hydrogen donation (Moniruzzaman, Sulaiman, Khalil, & Gan, 2013). In the present study, radical scavenging activity of honey was ascertained by the change in color from deep violet to light yellow on the addition of DPPH. A linear relationship was observed between the concentration of honey and antioxidant activity. Percent radical scavenging activity was found to be 23.76 and 54.76 at 20 mg/ml and 60 mg/ml of honey. Maximum radical scavenging activity (61.26%) was observed at 80 mg/ml of honey. Therefore, the antioxidant potential of natural, unpasteurized honey can be efficiently exploited in the treatment of disease that has a free radical origin. It is evident from this study that free radical scavenging compounds in honey may inhibit reactive oxygen species mediated cytotoxicity which is in accordance with Delhalle, Deregowski, Benoit, Merville, and Bours (2002).

3.2. Isolation and identification of bacterial strain

Three phenotypically different bacterial strains were isolated from honey. Of the three, only one isolate was subjected to further

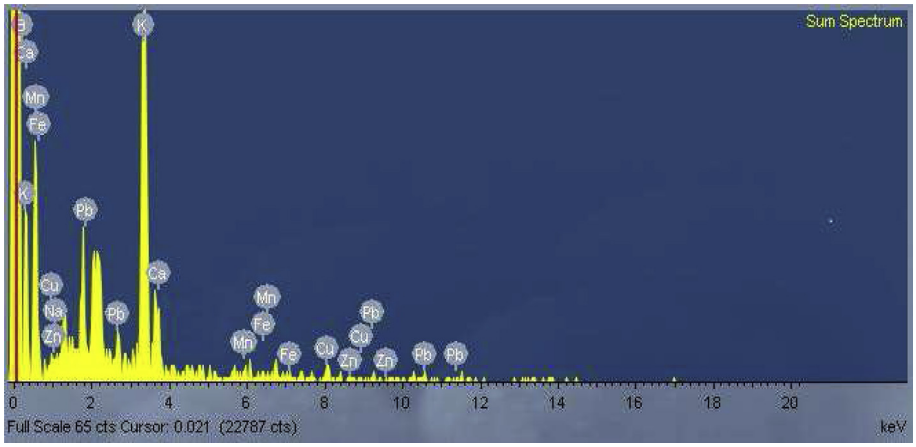

Fig. 1. EDAX spectrum indicating the presence of essential elements in natural honey.

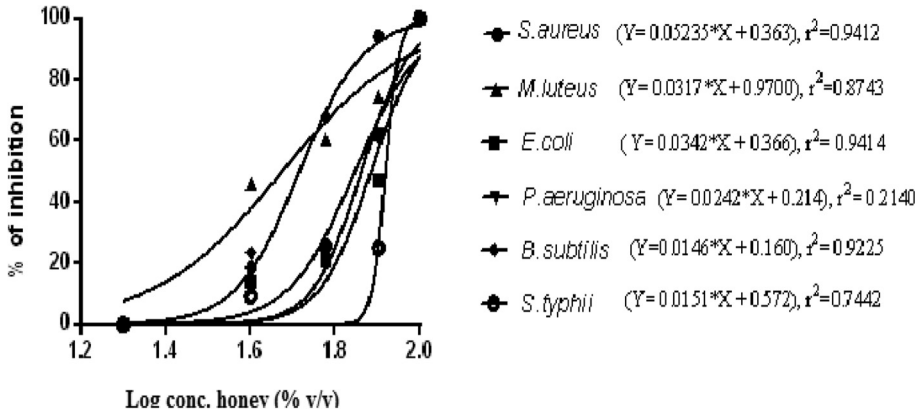

Fig. 2. Dose–response curve for the antibacterial activity of natural honey against different bacterial pathogens.

Table 2
Biochemical characteristics of *Gluconobacter oxydans* isolated from natural honey.

Biochemical tests	*Gluconobacter oxydans*
Colony morphology	Irregular
Color	Orange
Shape	Rod
Grams stain	Gram negative
Motility	Motile
Endospore	–
Catalase	+
Oxidase	–
Gelatin hydrolysis	–
H_2S production	–
Methyl red test	+
Voges – proskauer test	+
Citrate utilization	+
Indole production test	–
Starch hydrolysis	–
Lipase	+
Nitrate reduction	–
Hemolytic activity	–
Optimal temperature	25–30 °C
Optimal pH	3.5–6
Carbon source utilization	
Glucose	+
Fructose	+
Galactose	+
Sucrose	+
Maltose	+
Arabinose	–

analyses based on its non-hemolytic activity and siderophore production (CAS assay). Non-hemolytic activity ensures the safety of the bacterium to be used as a probiotics. Biochemical profile facilitated the taxonomic identification of the isolate as *Gluconobacter* spp. The isolate was gram negative, rod shaped bacterium, respond positively for methyl red, voges proskauer test, citrate utilization and lipase production (Table 2). 16S rRNA gene partial sequence (accession number CMMB 1234) of the isolate and phylogenetic analysis supports the biochemical identification. Phylogenetic tree generated by neighbor-joining method is shown in Fig. 3. Based on the sequence analysis and phylogenetic tree the isolate was confirmed as *Gluconobacter oxydans*. The dendrogram shows reliability of branching order based on bootstrap analysis. Lambert, Kersters, Gossele, Swings, and De Ley (1981) isolated 56 *G. oxydans* strains from honeybees in three different regions at Belgium. Occurrence of *Gluconobacter* spp. in honeybees, honey and with different kinds of sugary materials was reported earlier by Kappeng and Pathom (2009). The other two bacterial isolates were identified as *Pseudomonas* sps. and *Bacillus* sps. based on fluorescence and endospore formation respectively.

3.3. Probiotic activity

3.3.1. Acid and bile salt tolerance

The acid tolerance profile of *G. oxydans* is depicted in Table 3. The isolate exhibited 100% survival at or above pH 5. Percentage survival of the isolate at pH 2 and pH 4 after 3 h of exposure was

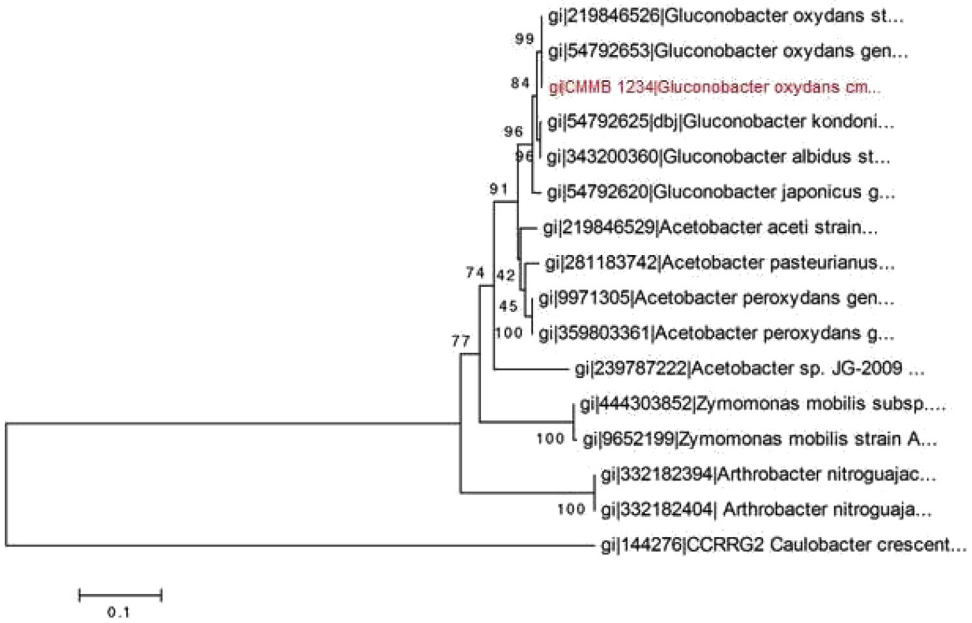

Fig. 3. Phylogenetic analysis of 16S rRNA gene sequences of *Gluconobacter oxydans*.

found to be 54.83 and 90.78 respectively. However, the isolate failed to survive at pH 1. Statistical analysis of the data using two-way ANOVA revealed that influence of pH and incubation time on growth of the *G. oxydans* was found to be significant ($p < 0.05$). The results establish the possibility of the strain to survive under acidic conditions that exist in the human gut (pH 2–5). In this study, the strains were exposed to different pH for 3 h because the transit time of the food along the human gut will be to a maximum of 3 h as reported by Jacobsen et al. (1999).

Bile salts are reported to inhibit bacterial growth by disrupting cell membrane. However, in the present study there was no great variation in the tolerance pattern of *G. oxydans* to different concentrations of bile salt. *G. oxydans* was found to tolerate 2% bile salt concentration. Tolerance to bile salt (2%) enables a probiotic strain to survive, grow and exert its action during gastrointestinal transit (Argyri et al., 2013).

3.3.2. Cholesterol assimilation assay

Cholesterol assimilation by the isolate at different time intervals is presented in Fig. 4. Cholesterol assimilation was found to be parallel with the growth (in terms of microgram protein per milliliter) of the isolate. It is evident from the result that cholesterol assimilation by *G. oxydans* increased with incubation time upto 48 h and declined thereafter. Gilliland, Nelson, and Maxwell (1985) observed a significant relationship between cholesterol assimilation and the degree of bile deconjugation in probiotics. It can be presumed that under acidic conditions probiotic bacteria destabilize cholesterol micelles and facilitate their co-precipitation with deconjugated bile salts.

Table 3
Influence of pH on the viable count and percent survival of *Gluconobacter oxydans* (average mean of three replicates ± standard error).

pH	Absorbance at 620 nm				Survival rate after 180 min (%)
	0 min	60 min	120 min	180 min	
1	0.07 ± 0.01	0.02 ± 0.00	0 ± 0.00	0 ± 0.00	0.0
2	0.31 ± 0.02	0.26 ± 0.03	0.21 ± 0.03	0.17 ± 0.03	54.83
3	0.64 ± 0.04	0.56 ± 0.07	0.54 ± 0.05	0.49 ± 0.04	76.56
4	0.76 ± 0.05	0.73 ± 0.06	0.7 ± 0.06	0.69 ± 0.07	90.78
5	0.84 ± 0.06	0.83 ± 0.031	0.83 ± 0.03	0.83 ± 0.031	100

SEm1 0.038, SEm2 0.034 and SEm3 0.078.
cd1 at 5% 0.078, cd2 at 5% 0.07, cd3 at 5% 0.15 and cv 19.87.
SEm; standard error of means, cd; Critical difference, cv; Coefficient of variance, number 1, 2 & 3 represents value of pH, incubation time and interaction between pH and incubation time.

3.3.3. Auto-aggregation, solvent adhesion and antibiotic susceptibility assay

Ability to adhere to the epithelial cells and mucosal surfaces was considered as a prerequisite for an ideal probiotics. Auto-aggregation percentage was found to be linearly associated with the incubation period (results not shown). Further, the isolate shows remarkable percent adhesion to xylene (2.03 ± 0.08), chloroform (8.67 ± 0.17) and ethyl acetate (6.03 ± 0.17). Relationship between auto-aggregation and adhesion ability to mucosal surfaces was reported earlier by Del Re et al. (2000). *G. oxydans* was found to be susceptible to tetracycline, kanamycin, gentamycin, and erythromycin but resistant to penicillin and rifampicin. Earlier studies

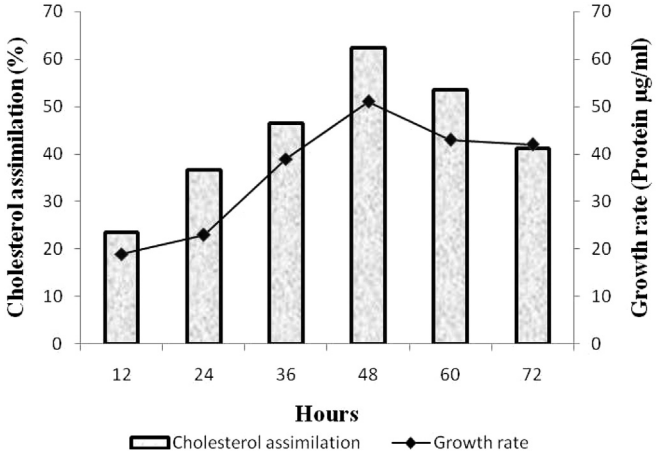

Fig. 4. Growth and Cholesterol assimilation of *G. oxydans* as a function of time.

Table 4
Classification and ligand denticity of hydroxamate siderophore produced by *G. oxydans*.

Isolate	λ max (nm) of ferrate siderophore		λ max (nm)	Color of the ferrate siderophores in electrophoresis	Inference	Color of the ferrate hydroxamate	Binding properties
	pH	λ max (nm)					
G. oxydans	4	403	8	Red	Trihydroxamate	Red	Hexadentate
	5	400					
	6	400					
	7	407					
	8	408					
	9	406					

Table 5
Growth promotion activity of siderophore produced by *G. oxydans*.

Isolates	Growth of siderophore auxotrophs after 24 h (μg/ml)	
	With siderophore	Without siderophore
Enterococcus spp	54 ± 2.89	19 ± 1.15
Bifidobacterium spp	51.6 ± 3.17	16.6 ± 2.60
Eubacterium spp	41.7 ± 2.90	11.7 ± 1.76
Bacillus clausi	45.6 ± 2.60	12.3 ± 2.60
Escherchia coli	13.6 ± 2.91	17.6 ± 3.18

Data are given as mean ± standard error of three replicates.

indicated that susceptibility to antibiotics may be an intrinsic property of the bacterium (Patel, Deshattiwar, Chaudhari, & Chincholkar, 2009).

3.4. Siderophore characterization

The positive results with $FeCl_3$ test, CAS assay and development of orange halos in CAS agar plate confirm siderophore production by *G. oxydans*. Cell free culture supernatant exhibits an absorbance maximum at 409 nm indicating the hydroxamate nature of the siderophore produced and this was confirmed by the positive Csaky test (results not shown). Raymond and Dertz (2004) reported that hydroxamates exhibit stronger binding affinity toward ferric iron than carboxylate and catecholate siderophores. In the present study, narrow shift in λ-max (up to 8 nm) with different pH and electrophoretic mobility results indicate trihydroxamate nature of the siderophore produced (Table 4). The binding property of the ligand with Fe^{3+} was determined by the retention of color by siderophore-metal complex. In the present study, the color of the ferric hydroxamate remained red over a wide pH range (4–9) indicating a hexadentate nature (Table 5). Baakza, Dave, and Dube (2004) reported that hexadentate trihydroxamates possess greater metal-binding constant than the corresponding mono hydroxamate complex.

3.4.1. Functional group determination by FTIR

IR spectrum of the purified siderophore extracted from *G. oxydans* (Fig. 5b) resembles the spectral data of desferrioxamine mesylate (Fig. 5a). Comparison of the functional groups confirms the hydroxamate nature of siderophore. The broad band at 3404.13 cm^{-1} represents the stretching vibration of OH group. A strong band at 1042.34 cm^{-1} represents the presence of primary aliphatic alcoholic group. Stretching vibration of aliphatic C–H

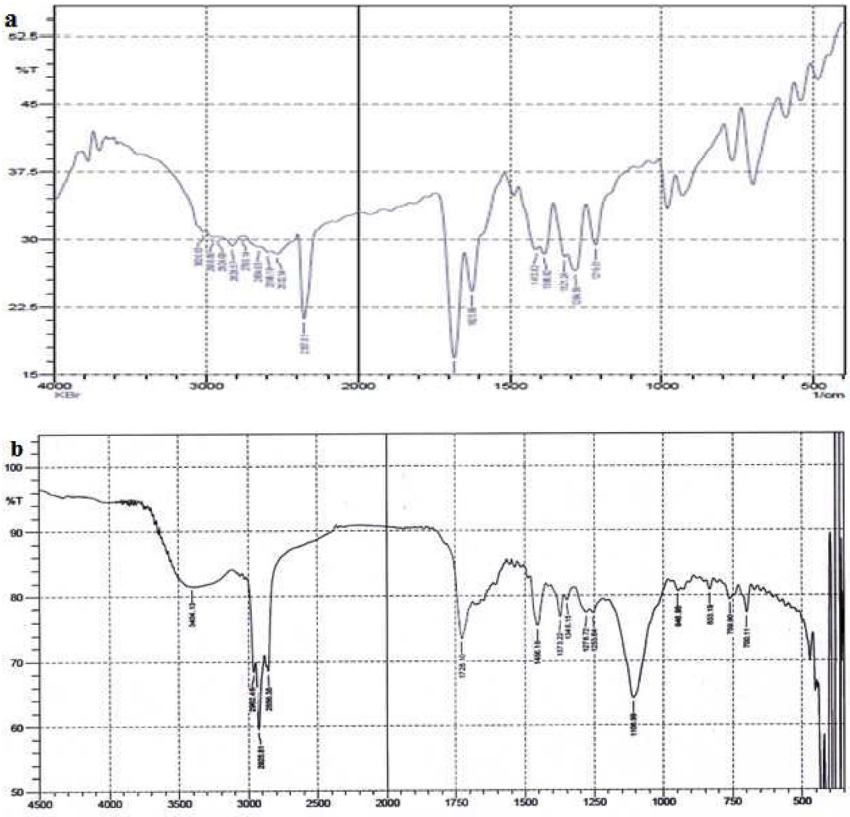

Fig. 5. a Infrared spectrum of desferrioxamine mesylate (Source: Murugappan, Karthikeyan, Aravinth, & Alamelu, 2012). b Purified siderophore extracted from *G. oxydans*.

group is represented by the bands in the region 2925.81 cm^{-1} – 2856.38 cm^{-1}. The band at 1728.10 cm^{-1} indicates the presence of C=O stretch. Multiple peaks in the range of 1250.21 cm^{-1} – 1450.99 cm^{-1} are characteristic of C–N stretch and C–H bend. Freibach, Yariv, Lapides, Hadar, and Chen (2005) indicated the presence of similar functional groups in desferrioxamine B.

3.4.2. Siderophore-mediated growth promotion

It is evident from the present investigation that intestinal bacterial strains grow better in the presence of siderophores than in pure culture (Table 5). Growth promotion activity of siderophores with auxotrophic strains under iron limited conditions confirms its function as an effective iron chelator. Ratledge and Dover (2000) reported that some enteric organisms meet their iron requirement by utilizing the siderophores produced by closely related organisms. In the present study, most of the intestinal bacterial strains tested were able to utilize the siderophores of *G. oxydans*. The negative result with *E. coli* indicates the absence of a corresponding siderophore receptor and its inability to grow under iron limited conditions. Harrison, Browning, Vos, and Buckling (2006) termed siderophore cross-utilizing organisms as “cheats” which pay none of the costs for production, but utilize the siderophores produced by their neighbors for iron chelation.

4. Conclusions

Considering the impressive list of health promoting benefits, honey and its innate microflora can be efficiently exploited as a nutraceutical and potential probiotics respectively. Anti-cholesteremic effect of *G. oxydans* is considered as one of the highly desirable probiotic traits. Furthermore, acid and bile tolerance property reveals the potential of the strain to survive under inhospitable environment of the human gastrointestinal tract. To the best of our knowledge this study is first to report siderophore production in probiotic *G. oxydans*. Cross feeding experiment reveals that exogenous siderophore induce growth of siderophore auxotrophic intestinal bacteria. Therefore, honey the natural food supplement is perceived as “safe” for the prophylaxis or alleviation of gastrointestinal disorders. In furtherance of this work, greener synthesis of silver (Ag) nanoparticles using honey as a reducing and capping agent for therapeutic applications is in progress.

Acknowledgments

Authors thank the college management for providing necessary facilities. Financial support from University Grants Commission (UGC) (UGC-MANF-MUS-TAM-5524- F1-17.1/2010 UGC-RGNF Ref No: F.14-2/(SC)/2010 MoEF-19-110/2008-RE) under Rajiv Gandhi National Fellowship (RGNF), Maulana Azad National Fellowship programme (MANF) (MANF-MUS-TAM-5524-F1-17.1/2010) and Ministry of Environment and Forest (MoEF) (MoEF-19-110/2008-RE) is greatly acknowledged. SASTRA University, Tamil Nadu is acknowledged for EDAX analysis.

References

Ahire, J. J., Patil, K. P., Chaudari, B. L., & Chincholkar, S. B. (2011). A potential probiotic culture ST2 produces siderophore 2,3- dihydroxybenzoylserine under intestinal conditions. *Food Chemistry, 127*, 387–393.

Ajibola, A., Chamunorwa, J. P., & Erlwanger, K. H. (2012). Long-term dietary supplementation with natural honey does not predispose growing male rats to metabolic syndrome. *BMC Proceedings, 6*, 1–2.

Akoh, D. A. (1991). Chemical analysis of honeys sugars and preserves. In *Manual of chemical analyses of food Product designed for National Agency for Food Drug Administration and Control (NAFDAC)* (pp. 87–90).

Argyri, A. A., Zoumpopoulou, G., Karatzas, K. A. G., Tsakalidou, E., Nychas, G. J. E., Panagou, E. Z., et al. (2013). Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. *Food Microbiology, 33*, 282–291.

AOAC. (1990). In K. Helrich (Ed.), *Official methods of analysis*. USA: Association of official Analytical Chemists: Inc.

Baakza, A., Dave, B. P., & Dube, H. C. (2004). Chemical nature, ligand denticity and quantification of fungal siderophores. *Indian Journal of Experimental Biology, 42*, 96–105.

Basualdo, C., Sgroy, V., Finola, M. S., & Marioli, J. M. (2007). Comparison of the antibacterial activity of honey from different provenance against bacteria usually isolated from skin wounds. *Veterinary Microbiology, 12*, 375–381.

Bilige, M., Liu, W., Rina, W., Wang, L., Sun, T., Wang, J., et al. (2009). Evaluation of potential probiotics properties of the screened Lactobacilli isolated from home-made koumiss in Mongolia. *Annals of Microbiology, 59*, 493–498.

Bogdanov, S., Martin, P., & Lullmann, C. (1997). Harmonised methods of the European honey commission. *Apidologie, 28*, 1–59.

Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Brenner, D. J., Krieg, N. R., & Staley, J. T. (2005). The Proteobacteria. In G. M. George (Ed.), *Bergey's manual of systematic bacteriology* (pp. 491–545). New York: Springer pub.

Cappucino, J. G., & Sherman, N. (2006). *Microbiology: A laboratory manual* (3rd ed.). Redwood City, California: The Benjamin/Cummings Publishing Company: Inc.

Cervanetes, M. A. R., Novelo, S. A. G., & Duch, E. S. (2000). Effect of the temporary hermic treatment of honey on variation of the quality of the same during storage. *Apiacta, 35*, 162–170.

Codex Alimentarius. (2001). Draft revised standard for standard for honey. *Alinorm, 25*, 19–26.

Del Re, B., Sgorbati, B., Miglioli, M., & Palenzona, D. (2000). Adhesion, autoaggregation and hydrophobicity of 13 strains of Bifidobacterium longum. *Letters of Applied Microbiology, 31*, 438–442.

Delhalle, S., Deregowski, V., Benoit, V., Merville, M. P., & Bours, V. (2002). NF-kB – dependent mnSOD expression protects adenocarcinoma cells from TNFα-induced apoptosis. *Oncogene, 21*, 3917–3924.

Erkkila, S., & Petaja, E. (2000). Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. *Meat Science, 55*, 297–300.

Ferreira, I. C. F. R., Aires, E., Barreira, J. C. M., & Estevinho, L. M. (2009). Antioxidant activity of Portuguese honey samples: different contributions of the entire honey and phenolic extract. *Food Chemistry, 114*, 1438–1443.

Freibach, H. S., Yariv, S., Lapides, Y., Hadar, Y., & Chen, Y. (2005). Thermo-FTIR spectroscopic study of the siderophore ferrioxamine B: spectral analysis and stereochemical implications of iron chelation, pH and temperature. *Journal of Agricultural and Food Chemistry, 53*, 3434–3443.

Gilliland, S. E., Nelson, C. R., & Maxwell, C. (1985). Assimilation of cholesterol by Lactobacillus acidophilus. *Applied Environmental Microbiology, 49*, 377–381.

Gulfraz, M., Iftikhar, F., Imran, M., Zeenat, A., Asif1, S., & Shah, I. (2011). Compositional analysis and antimicrobial activity of various honey types of Pakistan. *International Journal of Food Science & Technology, 46*, 263–267.

Hamouda, H. M., & Abouwarda, A. (2011). Antimicrobial activity of bacterial isolates from honey. *International Journal of Microbiological Research, 2*, 82–85.

Harrison, F., Browning, L. E., Vos, M., & Buckling, A. (2006). Co-operation and virulence in acute Pseudomonas aeruginosa infections. *BMC Biology, 4*, 21.

Jacobsen, C. N., Rosenfeldt, N. V., Hayford, A. E., Moller, P. L., Michaelsen, K. F., & Paerregaard, A. (1999). Screening of probiotic activities of forty seven strains of *Lactobacillus spp*. by in vitro techniques and evaluation of colonization ability of five selected strains in human. *Applied Environmental Microbiology, 65*, 4949–4956.

Jalal, M. A. F., & Helm, D. (1990). Isolation and spectroscopic identification of fungal siderophores. In G. Winklemann (Ed.), *Handbook of microbial iron chelates* (pp. 235–269). Bacon Raton, FL, USA: CRC Press.

Kahraman, T., Buyukunal, S. K., Vural, A., & Altnatmaz, S. S. (2010). Physico-chemical properties in honey from different regions of Turkey. *Food Chemistry, 123*, 41–44.

Kamboj, R., Bera, M. B., & Nanda, V. (2013). Evaluation of physico-chemical properties, trace metal content and antioxidant activity of Indian honeys. *International Journal of Food Science & Technology, 48*, 578–587.

Kappeng, K., & Pathom, W. (2009). Isolation of acetic acid bacteria from honey. *International Journal of Science and Technology, 3*, 71–76.

Lambert, B., Kersters, K., Gossele, F., Swings, J., & De Ley, J. (1981). *Gluconobacters* from honey bees. *Antonie van Leeuwenhoek, 47*, 147–157.

Moniruzzaman, M., Sulaiman, S. A., Khalil, M. I., & Gan, S. H. (2013). Evaluation of Physicochemical and antioxidant properties of sourwoon and other malaysian honeys: a comparison with manuka honey. *Chemistry Central Journal, 7*, 138.

Murugappan, R. M., Karthikeyan, M., Aravinth, A., & Alamelu, M. R. (2012). Siderophore-mediated iron uptake promotes yeast-bacterial symbiosis. *Applied Biochemistry and Biotechnology, 168*, 2170–2183.

Naman, M., Faid, M., & El-Adlouni, C. (2005). Microbiological and physico-chemical properties of Moroccan honey. *International journal of Agricultural Biology, 5*, 773–776.

Patel, A. K., Deshattiwar, M. K., Chaudhari, B. L., & Chincholkar, S. B. (2009). Production, purification and chemical characterization of the catecholate siderophore from potent probiotic strains of Bacillus spp. *Bioresource Technology, 100*, 368–373.

Payne, S. N. (1994). Detection, isolation and characterization of siderophores. *Methods of Enzymology, 235*, 329–344.

Ratledge, C., & Dover, L. G. (2000). Iron metabolism in pathogenic bacteria. *Annual Review of Microbiology, 54*, 881–941.
Raymond, K. N., & Dertz, E. A. (2004). Biochemical and physical properties of siderophores. In J. H. Crosa, A. R. Mey, & S. M. Payne (Eds.), *Iron transport in bacteria* (pp. 3–17). Washington, DC: ASM Press.
Saarela, M., Mogensen, G., Fonden, R., Matto, J., & Mattila-Sandholm, T. (2000). Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology, 84*, 197–215.
Sayeed, R. Z., & Chincholkar, S. B. (2006). Purification of siderophores of *Alkaligenes faecalis* on Amberlite XAD. *Bioresource Technology, 97*, 1026–1029.
Schwyn, B., & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry, 160*, 47–56.
Silva, A. D. S., Alves, C. N., Fernanes, K. D. G., & Muller, R. C. S. (2013). Classification of honeys from Pará state (Amazon region, Brazil) produced by three different species of bees using chemometric methods. *Brazilian Journal of Chemical Society*, 1–11.
Suvarna, V. C., & Boby, V. U. (2005). Probiotics in human health: a current assessment. *Current Science, 88*, 1744–1748.
Tsekoura, F., Alexopoulos, A., Stefanis, C., Papadopoulos, I., Voidarou, C., Bolkas, S., et al. (2006). Aerobic and anaerobic bacteriology of Greek honeys. In *Proceedings of the International Congress of Bioprocessing in Food Industry (ICBF)*. Patras-Greece.
Voidarou, C., Alexopoulos, A., Plessas, S., Karapanou, A., Mantzourani, I., Stavropoulou, E., et al. (2011). Antibacterial activity of different honeys against pathogenic bacteria. *Anaerobe*, 1–5.
Winkelmann, G., Schmid, D. G., Nicholson, G., Jung, G., & Colquhoun, D. J. (2002). Bisucaberin-A dihydroxamate siderophore isolated from *Vibrio salmonicida*, an important pathogen of farmed Atlantic salmon (Salmo salar). *Biometals, 15*, 153–160.